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Structural Elements Required for Replication and Incompatibility of the *Rhizobium etli* Symbiotic Plasmid

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The symbiotic plasmid of *Rhizobium etli* CE3 belongs to the RepABC family of plasmid replicons. This family is characterized by the presence of three conserved genes, *repA*, *repB*, and *repC*, encoded by the same DNA strand. A long intergenic sequence (*igs*) between *repB* and *repC* is also conserved in all members of the plasmid family. In this paper we demonstrate that (i) the *repABC* genes are organized in an operon; (ii) the RepC product is essential for replication; (iii) RepA and RepB products participate in plasmid segregation and in the regulation of plasmid copy number; (iv) there are two *cis*-acting incompatibility regions, one located in the *igs* (*inc*α) and the other downstream of *repC* (*inc*β) (the former is essential for replication); and (v) RepA is a *trans*-acting incompatibility factor. We suggest that *inc*α is a *cis*-acting site required for plasmid partitioning and that the origin of replication lies within *inc*β.

Rhizobium etli, like other members of the genus, contains large, low-copy-number plasmids. One of these, the symbiotic plasmid (pSym), carries many of the genes required for the formation of the nitrogen-fixing nodules which characterize the interaction of this bacterium with bean plants. pSym coexists in the same cell with 1 to 10 undercharacterized plasmids also known as "cryptic plasmids." Nevertheless, genes implicated in the synthesis of cellular components or in the utilization of nutrients have been located on some of these plasmids (14, 27).

Several basic replicons of *Rhizobium* and *Agrobacterium* plasmids have been cloned and sequenced: the *Agrobacterium tumefaciens* pTiB6S3, pTiC58, and pTi-SAKURA tumor-inducing plasmids (24, 41, 42); the *Agrobacterium rhizogenes* pRiA4b root-inducing plasmid (31); the *Rhizobium leguminosarum* pRL8JI cryptic plasmid (43); the *R. etli* p42d symbiotic plasmid (36); the *Rhizobium* sp. pNGR234a symbiotic plasmid (11); and the *Sinorhizobium meliloti* pRmeGR4a plasmid (28). The first seven plasmids belong to the RepABC family and share the same genetic organization and a high degree of sequence identity in the three genes that identify these plasmids. Plasmid pRmeGR4a belongs to the RepC family, which is related to the former because they share a high degree of sequence identity with the *repC* gene, but *repA* and *repB* are not present. It has been shown that plasmids of the RepC family are common in field populations of *Rhizobium* (7, 37, 44).

Recently, a member of the RepABC plasmid family (pTAV320) was isolated from *Paracoccus versutus*, a bacterium unrelated to the *Rhizobiaceae* family, thus raising the possibility of finding this type of plasmid in other α proteobacteria (4).

Three genes, *repA*, *-B*, and *-C*, are required for the stable replication and adequate partitioning of this plasmid family. The three genes are located in the same DNA strand and in the same order in all members of the family. The RepA and RepB products are similar to proteins involved in the partitioning of plasmids F and P1 (46). Moreover, mutations in *repA* or *repB* of pTiB6S3 affect plasmid stability. It has been suggested that the products of these genes act as replication enhancers (42), but the data presented do not contradict a role in segregation (8). Also, it has been shown that RepC is the principal initiation protein, since frameshift mutations within the *repC* gene completely abolish replication functions (42). Despite the high degree of sequence identity that they share, plasmids pTiB6S3 and pRiA4b are compatible, and the Rep proteins of the first plasmid are not interchangeable with the corresponding products of the second in complementation tests. This indicates that the Rep proteins are highly specialized and specific (42). A large intergenic sequence (*igs*) is found between *repB* and *repC* in all replicators of the family.

Members of the RepABC plasmid family are unit copy plasmids or very low copy number plasmids. However, sequence analysis has shown that these plasmids do not contain DnaA boxes, at least with the signatures proposed by Schaper and Messer (39) and by Fuller et al. (12), and do not contain repeated sequences (iterons), which are common themes in low-copy-number plasmids.

Incompatibility has been detected between *Rhizobium* plasmids and between *Rhizobium* and *Agrobacterium* plasmids (5, 20, 21, 34, 35). The symbiotic plasmids, unrelated to their host range determinants, can belong to different incompatibility groups (16, 19). Nevertheless, the molecular basis for *Rhizobium* plasmid incompatibility is poorly understood.

The basic replicon of the symbiotic plasmid (p42d) of *R. etli* CE3 belongs, as mentioned above, to the RepABC plasmid family. This basic replicon is contained within a 5.6-kb *Hin*dIII fragment and confers replication stability on a plasmid normally incapable of replicating in *R. etli* (pSUP202). A recombinant plasmid containing the 5.6-kb *Hin*dIII fragment introduced into a *recA* derivative of CE3 exhibited incompatibility with p42d and replicated with the same copy number as the symbiotic plasmid. These data indicate that all the sequences of the symbiotic plasmid required for replication, copy number control, stability, and incompatibility reside in this fragment (36). With the aim of elucidating the molecular basis of the incompatibility and replication functions of plasmids of the

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RepABC family, we report here the identification of the *trans*-acting elements and the *cis*-acting sites required for incompatibility and their relation to replication and segregation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. *Rhizobium* strains were grown at 30°C in PY medium (32). Antibiotics were added at the following concentrations (in micrograms per milliliter): nalidixic acid, 20; tetracycline, 10; kanamycin, 30; chloramphenicol, 25; and ampicillin, streptomycin, or spectinomycin, 100 or as otherwise indicated.

Bacterial matings. pSUP202 and pRK7813 derivatives were introduced into *Rhizobium* using pRK2013 as the helper plasmid. Strains were grown in the proper liquid medium to stationary phase, mixed in a proportion (donor-helper) of 2:1 on PY plates, and incubated at 30°C overnight. The cells were resuspended in fresh PY medium, and serial dilutions were plated on the appropriate selective medium.

Plasmid profiles. Profiles of high-molecular-weight plasmids were obtained by the in-gel lysis procedure described by Wheatcroft et al. (45).

DNA isolation, manipulation, and hybridization. Genomic DNA was isolated by employing the components and instructions of the DNA-RNA isolation kit (Amersham). Plasmid DNA was isolated as described by Sambrook et al. (38). DNAs were restricted and ligated under the conditions specified by the enzyme manufacturer (Amersham). *Taq* polymerase or elongase (Gibco BRL) was used for PCR. The PCR products were cloned using a pMOSblue T vector kit or a pMOSblue blunt-ended vector kit (Amersham). DNA restriction fragments were separated for hybridizations by electrophoresis in 1% agarose gels, transferred onto Hybond N+ membranes (Amersham), and cross-linked in a UV cross-linker unit (Stratagene).

Hybridizations were performed overnight using [α^{32}P]dCTP-labeled probes (Megaprime kit; Amersham) under high-stringency conditions (65°C in rapid-hyb buffer [Amersham]). Hybridization signals were detected on X-OMAT-K films (Kodak) in the presence of intensifying screens or in a PhosphorImager (Molecular Dynamics).

Plasmid stability. Plasmid stability was calculated according to the procedure described by Durland and Helinski (10). Briefly, stationary-phase cultures were diluted in fresh medium without selection to give an initial optical density of 0.001 at 620 nm and cultivated for 9, 18, and 31 generations. Samples taken at these times were serially diluted and plated onto solid medium in the absence of selective drugs. One hundred colonies were chosen and picked onto plates with and without the selective antibiotic.

Plasmid construction. To identify the elements required for incompatibility and a stable replication of pH3, two collections of subclones, PCR products, and deletion derivatives of pHY were created. One collection, dedicated to identifying elements involved in replication, was constructed in the mobilizable vector pSUP202. This plasmid is unable to replicate in *Rhizobium*. All members of this collection were named with the prefix pRE- followed by the name of the insert. The second collection, made with the aim of identifying incompatibility determinants, was constructed in pRK7813, a vector capable of replicating in *R. etli* (22). The members of this collection were named with the prefix pKRE- and the name of the insert. A description of the construction of each plasmid is given in Table 1, and a scheme of their construction is shown in Fig. 1.

Plasmid incompatibility. To determine the incompatibility of the pKRE derivatives, the plasmids were introduced into CFNX101. The plasmid profiles of at least four transconjugants from each cross were analyzed.

Plasmid replication in *R. etli*. To determine the replication capabilities of the pRE derivatives in *R. etli*, the plasmids were introduced into CFNX107. The plasmid profiles of at least four transconjugants of each cross were analyzed. A recombinant plasmid was considered to have the capability to replicate in *R. etli* if (i) the plasmid profile of the transconjugants showed a new band and hybridization with pSUP202 and (ii) the new plasmid could be recovered from the transconjugants by transformation or conjugation with *E. coli*.

Determination of plasmid copy number. Plasmid copy numbers of CFNX107 transconjugants containing the plasmids pH3, pRE-ΔA1, pRE-prepA-ΔB-C, and pRE-prepΔA-BC were evaluated as follows. Total DNA was isolated, digested with *Hin*dIII endonuclease, resolved in a 1% agarose gel, and transferred to Hybond N+ membranes (Amersham). The blot was then simultaneously hybridized with a 1.4-kb *Hin*dIII-*Eco*RI fragment of the chromosomally encoded gene *recA* and with a 1.38-kb PCR product of *repC*. The *recA* probe hybridized with a 1.9-kb fragment, and *repC* hybridized with a fragment ranging between 3.8 and 5.6 kb. Hybridization signals were quantified using a PhosphorImager SI (Molecular Dynamics). The plasmid copy number was calculated as the ratio of the integrated hybridization signal of *repC* (plasmid) and the integrated hybridization signal of *recA* (chromosome).

RESULTS

Replication and stability functions of the pH3 replicator region. The ability of plasmid pH3 to autonomously and stably replicate in *R. etli* and *A. tumefaciens* depends on its 5.6-kb *Hin*dIII insert. This insert contains three open reading frames homologous to the *repA*, *-B*, and *-C* genes found in other plasmids of the RepABC plasmid family (Fig. 1a). To identify the sequences essential for replication and stability, a set of subclones or deletion derivatives of the 5.6-kb *Hin*dIII insert were ligated into the nonreplicative vector pSUP202 and introduced into an *R. etli recA* strain lacking the symbiotic plasmid (CFNX107). As shown in Fig. 1b, plasmid pRE-MR (containing an insert with the complete *repABC* genes, including 270 bp upstream of *repA* and 500 bp downstream of *repC*) was the only plasmid able to replicate with the same stability as the parental plasmid, pH3. Plasmid pRE-MR contains the shortest insert having the same replicative properties as pH3, indicating that all elements required for stable replication reside within this insert. Plasmid pRE-ΔA1, a deletion derivative of pH3 which lacks most of the *repA* and *repB* genes, was able to replicate in CFNX107 but was rapidly lost in cultures without selective pressure (Fig. 2). This result indicates that at least one of these gene products is required for plasmid stability. To determine if both proteins participate in plasmid stabilization, two deletion-insertion derivatives of pRE-MR were constructed. The first one (pRE-prepΔA-BC) is an in-frame deletion-insertion of the *repA* gene in which a segment of 477 nucleotides has been replaced with a *Bam*HI site. The deleted segment includes the nucleotides encoding the ATP binding motif characteristic of this protein family (30). The second construct (pRE-prepA-ΔB-C) is an in-frame deletion-insertion of the *repB* gene in which a segment of 192 nucleotides has been replaced with a *Bam*HI site. Although these constructs are able to replicate in CFNX107, they were highly unstable, to an even greater extent than the *repAB* deletion derivative (Fig. 2). These results suggest that both the RepA and RepB products are required to promote stable replication.

To evaluate the role of RepC in replication, an ΩKm interposon was introduced into the *Bgl*II restriction site located within the *repC* gene of pH3. The mutant plasmid (pRE-repCΩKm) was unable to replicate in CFNX107, indicating that RepC is essential for replication.

The *repA*, *repB*, and *repC* genes are organized in a single operon. The *repA*, *-B*, and *-C* genes are encoded by the same DNA strand and contain two putative Shine-Dalgarno sequences, one located in the 5′ end of *repA* and the other in the 5′ end of *repC*, within the large intergenic sequence between *repB* and *repC*. This genetic arrangement suggests that the *repA*, *repB*, and *repC* genes are organized in a single operon. However, regions containing the *E. coli* σ^{70} promoter consensus [TTGACA(N_{17})TATAC/AA/T] were not found in the pH3 insert (17).

To determine if these genes are organized in a single operon, a genetic approach was followed. As mentioned above, *repA* and *repB* are not essential for replication while *repC* is indispensable for replication. As shown in Fig. 1b, a pSUP202 derivative (pRE-S1) containing the 3′ end of *repB* and the complete *repC* gene and its downstream region is unable to replicate in *R. etli*, indicating that *repC* lacks a promoter of its own. A pH3 derivative (pRE-ΔS1.2) lacking the 5′ end of *repA* and its upstream region is unable to replicate in *R. etli* as a result of a polar effect of this deletion on *repC*.

Plasmid pRE-MR replicates in *R. etli* as well as pH3, but the replacement of an internal fragment of *repA* or *repB* by an ΩKm cassette (pRE-repAΩKm and pRE-repBΩKm, respectively) eliminates the replication of these constructions, indicating a polar effect on *repC* (Fig. 1b). Together, these data indicate that the *repABC* genes are arranged in a single operon.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Reference
E. coli		
HB101	Host strain for plasmids	6
DH5α	Host strain for plasmids	18
R. etli		
CE3	Sm^r; nodulates *P. vulgaris*	32
CFNX101	*recA*::Ω Sp^r Sm^r; derivative of CE3	25
CFNX107	*recA*::Ω Sp^r Sm^r p42a$^-$ p42d$^-$; derivative of CE3	25
Plasmids		
pRK7813	Tc^r; RK2-based plasmid vector	22
pRK2013	Helper plasmid; Km^r	9
pSUP202	Ap^r Cm^r Tc^r; ColE1 origin; unable to replicate in *Rhizobium* spp.	40
pBSL128	Ap^r Km^r; contains an ΩKm cassette	2
pH3	pSUP202 derivative carrying a 5.6-kb *Hin*dIII fragment containing the basic replicon of p42d	36
pHY	pBluescript II SK(+) derivative carrying a 5.6-kb *Hin*dIII fragment containing the basic replicon of p42d	36
pRE-S1	pSUP202 derivative carrying a 2.4-kb *Sal*I-*Hin*dIII fragment	This work
pRE-ΔX1	pSUP202 derivative in which the *Xho*I internal fragment of the 5.6-kb *Hin*dIII insert was deleted (from nucleotide 3235 to 4170)[b]	This work
pRE-ΔA1	pSUP202 derivative in which two contiguous *Aat*II internal fragments of the 5.6-kb *Hin*dIII insert were deleted; the deletions eliminate a portion of the *repA* and *-B* genes (between nucleotides 1187 and 3124)	This work
pRE-ΔS1.2	pSUP202 derivative in which two contiguous *Sal*I internal fragments of the 5.6-kb *Hin*dIII insert were deleted; the deletion eliminates the 3′ end of the *repA* gene and its downstream region (between nucleotides 358 and 1314)	This work
pRE-MR	pSUP202 derivative containing the complete *repABC* genes; the segment was generated by PCR and carries nucleotides 793 to 5084	This work
pRE-prepΔA-BC	pSUP202 derivative containing the same insert as plasmid pRE-MR but with an in-frame deletion-insertion in the *repA* gene; the DNA sequence between nucleotides 1200 and 1667 inside the *repA* gene was replaced by a *Bam*HI restriction site; the insert was generated by ligating two PCR products in the correct direction; the first includes nucleotides 793 to 1200, and the second includes nucleotides 1667 to 5084	This work
pRE-prepA-ΔB-C	pSUP202 derivative containing the same insert as plasmid pRE-MR but with an in-frame deletion-insertion in the *repB* gene; the DNA sequence between nucleotides 2392 and 2586 inside the *repB* gene was replaced by a *Bam*HI restriction site; the insert was generated by ligating two PCR products in the correct direction; the first includes nucleotides 793 to 2392, and the second includes nucleotides 2586 to 5084	This work
pRE-Δincα	pSUP202 derivative containing the same insert as plasmid pRE-MR but with the intergenic sequence between *repB* and *repC* replaced by a *Bam*HI restriction site; the insert was generated by ligating two PCR products in the correct direction; the first encompasses nucleotides 793 to 3299, and the second includes nucleotides 3455 to 5084	This work
pRE-Δincβ	pSUP202 derivative containing the same insert as plasmid pRE-MR but in which the sequence downstream of *repC* was eliminated; the segment was generated by PCR and carries nucleotides 793 to 4660	This work
pRE-repCΩKm	pSUP202 derivative with the same insert as pH3 but in which the *repC* gene was interrupted by an ΩKm cassette (pBSL128 was the ΩKm cassette donor); the cassette was introduced by utilizing a *Bgl*II restriction site within the *repC* gene	This work
pRE-repBΩKm	pSUP202 derivative with the same insert as plasmid pRE-prepA-ΔB-C but with an ΩKm cassette inserted in the *Bam*HI site located within the remaining sequence of *repB* (pBSL128 was the ΩKm cassette donor)	This work
pRE-repAΩKm	pSUP202 derivative with the same insert as plasmid pKRE-prepΔA-BC but with an ΩKm cassette inserted in the *Bam*HI site located within the remaining sequence of *repA* (pBSL128 was the ΩKm cassette donor)	This work
pKRE-1	pRK7813 derivative with the same insert as pH3	This work
pKRE-prepAB	pRK7813 derivative containing the complete *repAB* genes; the segment was generated by PCR and carries nucleotides 793 to 3299	This work
pKRE-incβ	pRK7813 derivative with an *Eco*RI fragment of 502 bp (*inc*β)	This work
pKRE-incα	pRK7813 derivative containing the intergenic sequence between *repB* and *-C* genes (*inc*α)	This work
pKRE-S3	pRK7813 derivative with a 959-bp *Sal*I fragment, including the upstream region and 5′ end of *repA*	This work
pKRE-repAB	pRK7813 derivative containing the complete *repAB* genes but excluding the upstream region of *repA*; the segment was generated by PCR and carries nucleotides 1063 to 3299	This work
pKRE-repC	pRK7813 derivative containing the sequence corresponding to the open reading frame of *repC*	This work
pKRE-prepΔA-B	pRK7813 derivative containing a PCR product obtained from plasmid pRE-prepΔA-BC; the insert contains *repB* and the same deletion in the *repA* gene as plasmid pRE-prepΔA-BC but lacks *inc*α, *repC*, and *inc*β	This work
pKRE-prepA-ΔB	pRK7813 derivative containing a PCR product obtained from plasmid pRE-prepA-ΔB-C; the insert contains *repA* and the same deletion on the *repB* gene as plasmid pRE-prepA-ΔB-C but lacks *inc*α, *repC*, and *inc*β	This work
pKRE-prepA	pRK7813 derivative containing the complete *repA* gene, including the upstream region; the segment was generated by PCR and carries nucleotides 793 to 2277	This work

[a] Plasmid derivative descriptions use nucleotide numbers of the sequence of pH3 as coordinates.
[b] GenBank accession number U80928.

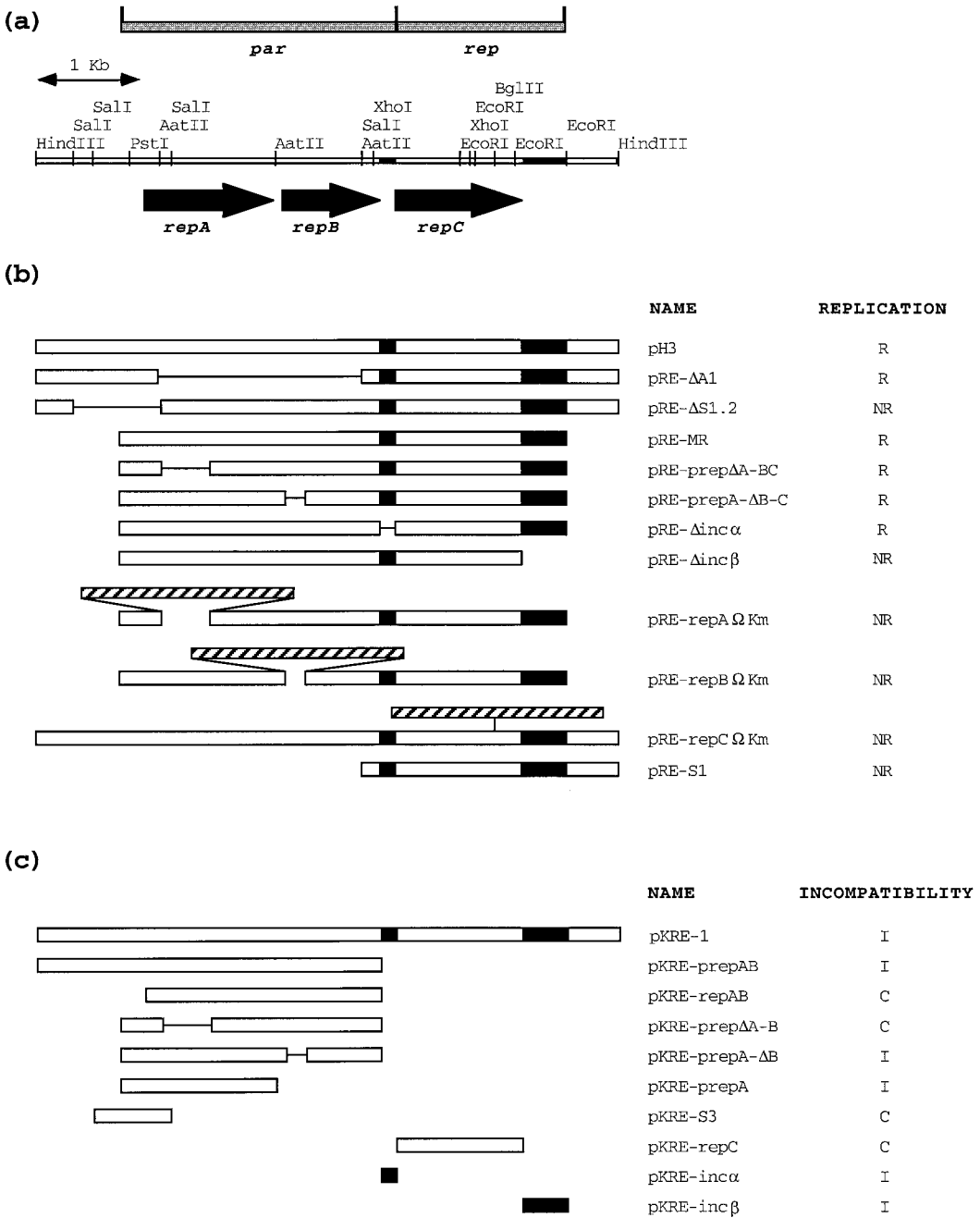

FIG. 1. Replication capabilities and incompatibility properties of pH3. (a) Restriction map of the pH3 insert. The shaded bar indicates regions involved in partition and replication. The orientations of the three open reading frames are indicated by arrows. The solid boxes indicate the locations of the *cis*-acting regions, which exhibit incompatibility with the symbiotic plasmid. (b) The open boxes represent the DNA inserts contained in each plasmid derivative. The thin lines connecting the open boxes indicate the DNA regions lost in the deletion derivatives. The hatched boxes represent ΩKm cassettes. In the column labeled "replication," R indicates that the plasmid derivative has the ability to replicate in the *R. etli* strain CFNX107, and NR indicates that the plasmid is unable to replicate in this strain. (c) The open boxes represent the DNA inserts contained in each plasmid derivative. The thin lines between the open boxes indicate the DNA regions lost in the deletion derivatives. In the column labeled "incompatibility," I indicates that the plasmid derivative is incompatible with pSym of *R. etli* strain CFNX101, and C indicates that the construct is compatible with pSym of *R. etli* strain CFNX101. The solid boxes indicate the positions of the DNA regions containing *cis*-acting incompatibility determinants.

RepA and RepB are involved in the control of plasmid copy number. Plasmid copy number is one factor that influences plasmid stability. Plasmid pH3 in *R. etli* has the same copy number (between one and two copies per chromosome) as its parental plasmid, pSym. However, plasmid derivatives lacking most of the *repA* and *repB* genes (pRE-ΔA1), or with an in-frame deletion of *repA* (pRE-prepΔA-BC) or *repB* (pRE-prepA-ΔB-C), are unstable. To determine if this instability is the result, at least in part, of a diminished plasmid copy number, the plasmid/chromosome ratios of strains CFNX107(pRE-ΔA1), CFNX107(pRE-prepΔA-BC), and CFNX107(pRE-prepA-ΔB-C) were determined (Fig. 3). Plasmid pRE-prepΔA-BC contained 3.1 ± 0.98 plasmid copies per chromosome, slightly more than pH3 (1.9 ± 0.09 copies per chromosome), and

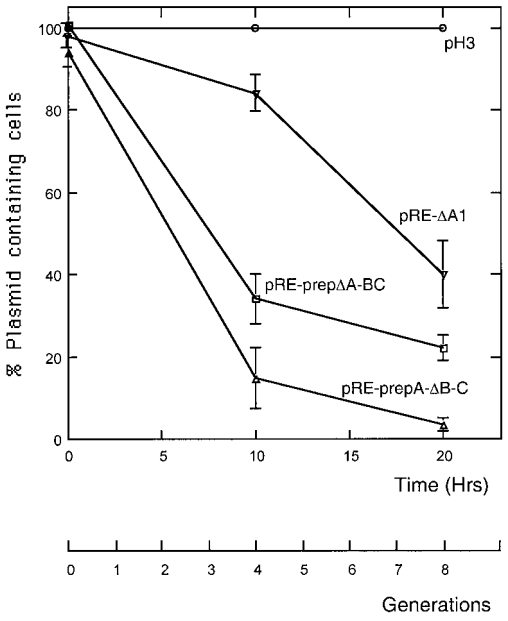

FIG. 2. Plasmid stability. Plasmid loss from populations of cells carrying different constructions with chloramphenicol resistance genes. ○, CFNX107 cells carrying plasmid pH3; □, CFNX107 cells carrying plasmid pRE-prepΔA-BC; ▽, CFNX107 cells carrying plasmid pRE-ΔA1; △, CFNX107 cells carrying plasmid pRE-prepA-ΔB-C. The graph shows the plasmid retention means (x) of three independent experiments ± the standard deviations.

plasmid pRE-prepA-ΔB-C contained 1 ± 0.02 copies per chromosome. Surprisingly, plasmid pRE-ΔA1 had a copy number (6.4 ± 0.04 plasmid copies per chromosome) three times higher than that of pH3. These results indicate that RepA and RepB are involved in the control of the plasmid copy number.

***cis*-acting sites required for incompatibility.** Incompatibility is defined as the inability of two different plasmids to reside in the same cell as independent replicons in the absence of selective pressure as a consequence of sharing similar replication and/or partition systems. To identify the incompatibility determinants present in pH3, several restriction fragments, PCR products, or deletion derivatives of the pH3 insert were subcloned in pRK7813 and introduced into CFNX101 (a *recA* derivative of the wild-type strain). Plasmid pRK7813 was chosen because it is a multicopy plasmid (two to seven copies per cell) capable of replicating in *R. etli* and because it is compatible with the six plasmids present in the *R. etli* wild-type strain. The incompatibility properties of these derivatives were evaluated by plasmid profile analysis. A DNA fragment was considered to exhibit incompatibility if its introduction into CFNX101 (i) caused the displacement of the symbiotic plasmid or (ii) induced the cointegration of the symbiotic plasmid with another cognate plasmid. The latter situation was interpreted as a result of the symbiotic plasmid's inability to survive as an independent replicon under selective pressure. A summary of the results is shown in Fig. 1c.

Plasmid pKRE-1 carrying the 5.6-kb *Hin*dIII fragment exhibits incompatibility with the symbiotic plasmid to the same extent as pH3, although its replication does not necessarily depend on itself. All constructs containing the intergenic sequence located between *repB* and *-C* or constructs harboring the 500-bp *Eco*RI fragment located immediately downstream of *repC* exhibited incompatibility with the symbiotic plasmid (Fig. 1c and data not shown). Moreover, a pRK7813 derivative harboring a PCR product containing no more than the intergenic sequence and a pRK7813 derivative containing only the 0.5-kb *Eco*RI fragment downstream of *repC* were incompatible with the symbiotic plasmid. These results clearly indicate that the replicator of the symbiotic plasmid contains two incompatibility regions, one located within the intergenic region between the *repB* and *-C* genes (*inc*α) and the other (*inc*β) located within the *Eco*RI fragment downstream of *repC*. Neither of these regions encodes any protein, suggesting that they are *cis*-acting sites for incompatibility and probably targets for proteins involved in replication and/or partitioning. It is important to point out that a pRK7813 derivative containing the promoter of the *repABC* operon (pKRE-S3) is unable to exhibit incompatibility with the symbiotic plasmid. Similarly, constructions carrying only the open reading frames of the *repA*, *-B*, and *-C* genes do not exhibit incompatibility with the symbiotic plasmid, indicating that these regions do not carry other *cis*-acting incompatibility regions.

RepA is a *trans*-acting element required for incompatibility. To test whether the RepA and/or -B products exhibit incompatibility with the symbiotic plasmid, these proteins were supplied in *trans* from a multicopy vector (pRK7813). Different constructs containing the *repAB* genes but lacking the *inc*α and *inc*β DNA regions were introduced into CFNX101. Transconjugant plasmid profiles were examined to determine incompatibility with the symbiotic plasmid. Figure 1c shows a scheme of the construction and a summary of the incompatibility results. Plasmid pKRE-prepAB, carrying the 270-bp *repA* upstream region and the complete *repA* and *-B* genes, displaced the symbiotic plasmid.

Plasmids containing only the *repA* upstream sequences or carrying only the *repA* and *-B* genes without the *repA* upstream region were unable to induce incompatibility with the symbiotic plasmid. Thus, the expression of the *repA* and/or *repB* gene is required to induce incompatibility. To determine if both or only one of the gene products was required for incompatibility, plasmids harboring (i) the *repA* gene with its upstream region (pKRE-prepA), (ii) the upstream *repA* region and the *repA* and *-B* genes but with *repA* containing an internal deletion (pKRE-prepΔA-B), and (iii) the upstream *repA* region and the *repA* and *-B* genes but with *repB* containing an internal deletion (pKRE-prepA-ΔB) were introduced into CFNX101. Plasmid profile analysis of the transconjugant showed that constructions containing the complete *repA* gene were incompatible with the symbiotic plasmid, indicating that RepA but not RepB is needed to induce incompatibility.

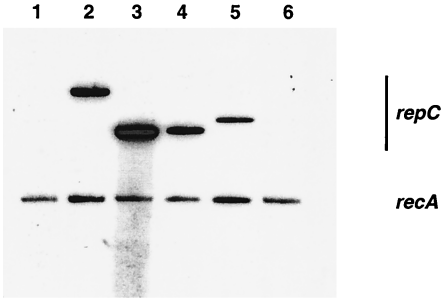

FIG. 3. Plasmid copy number. Autoradiogram of a Southern blot of total DNA digested with *Eco*RI and *Hin*dIII and probed simultaneously with a chromosomal detector (*recA*) and with a *repC* detector. The plasmid copy number of each strain was calculated as the ratio of the integrated hybridization signal of *repC* (plasmid) and the integrated hybridization signal of *recA* (chromosome). Lane 1, CFNX107; lane 2, CFNX107(pH3); lane 3, CFNX107(pRE-ΔA1); lane 4, CFNX107(pRE-prepΔA-BC); lane 5, CFNX107(pRE-prepA-ΔB-C); and lane 6, CFNX107.

The *cis*-acting incompatibility region and its relation with replication and/or partitioning. The *inc*α and *inc*β sites are potential targets for replication, partitioning, or regulatory proteins. Reasoning that the origin of replication is essential for plasmid existence but that the partition site and regulation sites are dispensable in the short term, we constructed plasmid derivatives containing the *repA*, *-B*, and *-C* genes but lacking the *inc*β region or the *inc*α intergenic sequence (pRE-Δincβ and pRE-Δincα, respectively) and crossed them with CFNX107. Transconjugants were exclusively obtained with derivatives lacking *inc*α and only when a low concentration (15 μg ml^{-1}) of the selective antibiotic was used. Plasmid profile analysis of these transconjugants showed the presence of the construct as an independent entity, indicating that the new plasmid was capable of autonomous replication but was highly unstable. These data indicate that *inc*β is essential for replication and that the intergenic sequence between the *repB* and *repC* genes (*inc*α) contains a sequence involved in the stable replication of the symbiotic plasmid (a possible interaction site for the RepA and/or RepB protein) (Fig. 1b).

DISCUSSION

The minimal DNA fragment that the symbiotic plasmid of *R. etli* CE3 requires for stable replication and incompatibility is 4.3 kb. This size lies within the range of the 5 kb reported for plasmid pRL8JI (43) and the 4.2 kb reported for pTiB6S3 (42), which are members of the RepABC plasmid family.

In this work, the roles of the proteins encoded by the basic replicon of the symbiotic plasmid were determined. RepC is essential for replication, because an insertion of a ΩKm cassette in the *repC* gene abolished the replication ability of pH3. In plasmids pTiB6S3 and pTAV230, members of the RepABC plasmid family, it was also demonstrated that RepC is the limiting factor for replication and, accordingly, RepC was considered the initiator protein (4, 42). Derivatives of pH3 lacking most of the *repA* and *repB* genes or carrying an in-frame deletion of *repA* or *repB* showed reduced stability, indicating that each of these genes has a role in the stability of the pSym basic replicon. A plasmid harboring the *repB* deletion is significantly less stable than the plasmid derivative with *repA* deleted, and this construction is less stable than a plasmid lacking most of the *repA* and *repB* genes. These findings can be interpreted in two ways: first, that these plasmid derivatives have defects in the segregation machinery, and second, that these plasmids have a reduction in the frequency of the initiation of replication. To choose between the two interpretations, the plasmid copy numbers of these deletion derivatives were determined and compared with the plasmid copy number of the parental plasmid (pH3). Plasmid derivatives with an in-frame deletion of *repA* had slightly higher copy numbers than the parental plasmid. On the other hand, a pH3 derivative with a deletion of *repB* has a plasmid copy number similar to that of pH3, indicating that the frequency of initiation is not reduced; thus, we concluded that RepA and RepB are part of the plasmid segregation machinery. Furthermore, RepA and RepB products are homologous to the proteins of the *sop*/*par* family of partition systems, and the relative positions of the genes coding for these products are similar to those of the *sop*/*par* family (46).

The best-studied members of the *sop*/*par* family are the partition systems of the F and P1 plasmids (*sop* and *par*, respectively). Each system consists of two polypeptides (A and B), encoded by a single operon, and a *cis*-acting site. The gene encoding A precedes that encoding B, and the *cis*-acting site (centromerelike site) is located immediately downstream of the B gene. Both proteins participate in the autoregulation of the operon and, together with the centromerelike site, in plasmid partitioning (46).

Genetic evidence indicates that the *repA*, *-B*, and *-C* genes are organized in a single operon: a pH3 deletion derivative lacking the 5′ end of *repA* and its upstream sequence was unable to replicate in *R. etli*, indicating that the promoter of the replicator genes is located upstream of *repA*. Moreover, insertion derivatives with ΩKm cassettes, but not in-frame deletions of *repA* or *repB*, were unable to replicate, indicating a polar effect of these insertions on *repC*. This is an unusual situation, in which genes implicated in partition and in replication are organized in the same operon.

Transconjugants containing plasmid pRE-ΔA1 contain more plasmid copies than transconjugants containing pH3 or plasmid derivatives with in-frame deletions of the *repA* or *repB* gene, suggesting that the *repA* and *repB* products act together to regulate the plasmid copy number. Currently, we are testing the simplest hypothesis, namely, that RepA and RepB repress operon transcription and consequently the quantity of RepC, the initiator protein, so that plasmids lacking the RepA and RepB products will be increased in copy number. An explanation for the increased stability observed for plasmid pRE-ΔA1 compared with that of the deletion derivatives of *repA* and *repB* is that the elevated copy number of pRE-ΔA1 partially compensates for defects in partition.

Plasmid pH3 exhibits incompatibility with the symbiotic plasmid when introduced into an *R. etli recA* strain. Two small DNA regions within the pH3 insert exhibited incompatibility when they were introduced into a replicable vector. One is located in the intergenic sequence between *repB* and *repC* (*inc*α), and the other is located within a 500-bp *Eco*RI fragment downstream of *repC* (*inc*β). Neither of these is a coding region, suggesting that they are *cis*-acting sites for partitioning and/or replication. A comparative sequence analysis of *inc*α and *inc*β did not show any obvious similarity between them, and repetitive sequences were not found within or between them. A possible explanation is that the factor(s) interacting with *inc*α is different from those interacting with *inc*β.

A functional origin of replication is an essential feature of a plasmid but, in the short term, the *cis*-acting partitioning site is dispensable. Our results showed that only plasmids lacking *inc*α were capable of replication, although they were very unstable. As *inc*β appears to be indispensable for plasmid replication and deletion of *inc*α produced a replicable but unstable plasmid, we tentatively conclude that the origin of replication resides within *inc*β and *inc*α is a *cis*-acting partitioning site. From this assumption it follows that the *cis*-acting partitioning site is located immediately downstream of *repB*, which is precisely the situation found in members of the *sop*/*par* partition system family. The *cis*-acting sites for partitioning of the P1 and F plasmids are also incompatibility determinants (3, 15, 33).

In contrast, for pTAV320, the most divergent member of the RepABC plasmid family, it was shown that it is possible to obtain transconjugants of a tetracycline-resistant construct containing the *repC* coding sequence under the control of the *lac* promoter in a strain lacking the parental plasmid (4). It was concluded that the origin of replication resides within the coding sequence of the *repC* gene. However, the *repC* coding region of pH3 does not exhibit incompatibility with the symbiotic plasmid, which would be expected if an origin of replication, controlled by an initiation protein, resided within the *repC* gene. This may indicate that the *repC* coding region contains an accessory origin of replication or that these two plasmids, despite their sequence homology, contain origins of rep-

lication located in different positions. To obtain a definitive answer, we are currently mapping the origin of replication of pH3 by two-dimensional agarose gel electrophoresis (26).

In plasmids F and P1, an overexpression of polypeptides A or B induces incompatibility as a result of abnormal DNA-protein complexes formed between the A and B polypeptides and their respective centromerelike DNA sequences or by the overrepression that the A and B products exert on the transcription of their respective operons (1, 8, 13, 23, 29). In the *R. etli* symbiotic plasmid, the RepA product was identified as a *trans*-acting incompatibility determinant, because the reintroduction of extra copies of the *repA* gene, under the control of its own promoter, caused displacement of the symbiotic plasmid. In contrast, extra copies of the *repB* gene did not exhibit incompatibility with the symbiotic plasmid. This behavior can be explained in a way similar to that for the F and P1 plasmids: (i) an excess of RepA forms an abnormal DNA-protein complex between the partition site and RepA and RepB or (ii) RepA is the principal repressor of the system, and thus, an excess of RepA blocks the transcription of the initiator protein and in this way induces incompatibility with the symbiotic plasmid. This experiment does not exclude the possibility that, by utilizing higher doses of RepB, an effect on incompatibility could be observed.

In summary, we have found that (i) RepC is essential for replication, (ii) the lack of *repA* and/or *repB* products destabilizes plasmid partitioning, (iii) the lack of the RepA and B products increases the plasmid copy number, (iv) the *repA*, *-B*, and *-C* genes are organized as an operon, (v) RepA is an incompatibility determinant, and (vi) plasmid pH3 contains two *cis*-acting incompatibility regions, one indispensable for replication (*inc*β) and the other dispensable in the short term but required for stability (*inc*α). We propose, as a working hypothesis, that (i) the RepA and -B products and their *cis*-acting site, *inc*α, are part of the segregation machinery of the symbiotic plasmid, (ii) RepC is the initiator protein and interacts with the origin of replication, probably located within *inc*β, and (iii) RepA and -B also act as repressors of the *repABC* operon and regulate the amount of RepC produced and, as a result, the rate of initiation of plasmid replication.

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